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**Hypochlorite Solution as a Decontaminant in Sulfur Mustard Contaminated Skin
Defects in the Euthymic Hairless Guinea Pig**

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ABSTRACT

Hypochlorite solutions are thought to be efficacious when used to topically decontaminate intact skin. However, few studies have examined the efficacy of decontamination of chemically contaminated wounds. Therefore, we compared the decontamination efficacy of sodium hypochlorite (0.5% and 2.5% solutions), calcium hypochlorite (0.5% and 2.5% solutions) and sterile water to untreated controls in wounds exposed to sulfur mustard (HD). Anesthetized euthymic hairless guinea pigs (EHGP) (n=6) were exposed to 0.4 LD₅₀ HD in a full-thickness 8 mm surgical biopsy skin defect (i.e., wound). Each animal was subsequently decontaminated, after a two-minute intra-wound exposure to liquid HD, with one of the decontamination solutions. Decontamination efficacy was determined by the visual grading of the HD-traumatized wound lesion and by comparison of the expected HD-induced leukocyte suppression. Leukocyte suppression was inconsistent in all animals; therefore, the visual grading was the only viable evaluation method. No significant differences were observed among wounds decontaminated with any of the solutions. However, the skin surrounding undecontaminated (but exposed) control animals showed the least visual pathology. The lesions induced following decontamination are presumed to be due to the mechanical flushing of HD onto the per-lesional skin, or by chemical damage induced by the solution, or HD-solution interaction. Further studies are required to best delineate the optimal decontamination process for HD contaminated wounds.

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INTRODUCTION

The U.S. Army Medical Department has had little experience in the management of chemically contaminated casualties since World War I.¹ Since that time, sulfur mustard (HD), a persistent vesicating agent, has persisted as a threat to the soldier. In the interim 75 years, chemical decontamination has remained the countermeasure of choice for loss reduction and lesion amelioration in the case where a lack of discipline, training, or luck have resulted in HD exposure.²

Many Chemical Warfare (CW) agents have been developed since World War I, and the Medical-Chemical Defense community has developed prophylactics and therapeutics to meet the threats. In the cases where specific countermeasures have not been developed, and in the interest of reducing morbidity of exposure, decontamination has been the hallmark of defense.

In 1917 chlorine bleach was the decontamination agent of choice to remove the chemical agents of concern, or to render them into harmless substances.¹ The succeeding 75 years of ongoing research for the perfect decontamination agent (i.e., an agent that reacts rapidly with the chemical agents, has little or no toxicity, is inexpensive, and does not cause excessive logistical burden)³ has resulted in the fielding of many products including: chlorine and non-chlorine based solutions, and ion-exchange resins.⁴ Present U.S. Army doctrine calls for the use of 0.5% hypochlorite solutions (i.e., dilute bleach solution), for wound and skin decontamination in cases of chemical exposure in a field environment.⁴ The decision for using these solutions was based on early experience in World War I, *in vitro* studies and limited subsequent *in vivo* experience thereafter.

Recent work⁵ has raised questions of the efficacy of hypochlorite solutions as decontaminating agents in contaminated wounds. As no definitive *in vivo* HD-exposed wound decontamination study has been found to definitively substantiate the use of hypochlorite solutions, our goal was to test the efficacy of several hypochlorite solutions in a animal model of an HD contaminated wound.

The euthymic hairless guinea pig (EHGP) has been extensively studied and accepted as the model of choice for HD study at our laboratory.^{6,7,8,9} Its unique features include a surface skin area which is uniformly graded and readily measurable in terms of gross and microscopic responses to HD vapor exposures.⁸ Marlow *et al* (1990) found that the EHGP produces microvesicles in response to cutaneous HD exposure; lesions substantially similar to those produced in man following similar exposure. Also, a recent study by one of the authors¹⁰ has revealed a temporal pattern of HD-induced leukopenia that can be used as a marker of total absorbed dose to determine efficacy of decontamination.

MATERIALS AND METHODS

ANIMALS

A total of 69 [Crl:IAF/HA(hr/hr)BR] euthymic hairless Hartley guinea pigs (EHGP) (*Cavia porcellus*), weighing between 500-1000 g, from the Newfield, NJ, breeding facility were used. Upon arrival they were maintained under an AAALAC accredited animal care and use program, quarantined and screened for evidence of disease before use. Guinea pigs were individually housed in plastic cages (Lab Products, Inc., Maywood, NJ) on compressed cellulose bedding (Cellu-dri, Shepherd Specialty Papers, Kalamazoo, MI), changed three times per week, and provided commercial certified guinea pig ration (Purina Mills, Inc., Richmond, IN) and tap water.

ad libitum. Animal holding rooms were maintained at $21 \pm 2^\circ\text{C}$ with $50 \pm 10\%$ relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air and maintained on a 12-hour light/dark full spectrum lighting cycle with no twilight.

SULFUR MUSTARD

Distilled sulfur mustard (HD) with a purity of 97.9-98.7% by nuclear magnetic resonance (NMR) was obtained from Chemical Research, Development and Engineering Center (CRDEC) to be used in this experiment. It has a density of 1.27 g/ml at 25°C and was stored unrefrigerated in a laminar flow hood during exposures.

WOUND AND EXPOSURE

The EHGP's were anesthetized by parenteral injection of xylazine (5 mg/kg) (Rompun[®], Haver Lockhart Laboratories, Box 390, Shawnee, KS 66201) and ketamine hydrochloride (75 mg/kg) (Ketaset[®], Bristol Laboratories, P.O. Box 657, Syracuse, NY 13201)¹¹ into the quadriceps musculature with a 25 gauge, 5/8 inch needle. Following induction into a surgical plane of anesthesia, a circular full-thickness skin segment was extracted from the mid-dorsal interscapular region by use of a sterile 8 mm skin biopsy punch (Bakers Biopsy Punch, Baker/Cummins, Division of Key Pharmaceuticals, Inc., Miami, FL), protecting underlying tissues from trauma by manually "tenting" the skin prior to excision. Subsequently, the animals were placed in ventral recumbency for HD exposure/decontamination while still under anesthesia.

Sulfur mustard, at a dose of 20 mg/kg (0.4 LD_{50}^{10}), was applied into the resultant skin defect of the EHGP with a Hamilton microliter syringe (Hamilton Co., Reno, NV) and 22 gauge 1 inch needle (Becton, Dickinson & Co., Columbus, NE). This HD dose was contained within the iatrogenic skin defect by careful animal positioning in ventral recumbency. Every attempt was made to ensure that an accurate dose was delivered, and HD did not run from the wound. The animals were kept in a laminar flow hood for approximately 24 hours following exposure/decontamination, and subsequently removed to an animal holding room as outlined above.

DECONTAMINATION

SOLUTIONS

Sodium hypochlorite solutions were prepared by dilution from a stock solution of 4.0 to 6.0% purified grade sodium hypochlorite solution (Fisher Chemical, Pittsburgh, PA) to yield solutions of 0.5 ($\pm 0.1\%$) and 2.5% ($\pm 0.1\%$) activity. The pH levels of these solutions were within the range of 8.0 and 10.0.

Calcium hypochlorite solutions were prepared by dilution of reagent grade (65% available chlorine) calcium hypochlorite with HPLC water to obtain one-liter quantities of 0.5 and 2.5% calcium hypochlorite solution (also of basic pH).

Each solution was assayed for hypochlorite concentration by the iodometric titration method and stored in sealed containers (Nalgene, Rochester, NY) under refrigeration prior to use (within 24 hours).

PROCEDURE

Animals were decontaminated two minutes post-exposure by direct contact of the

various decontamination solutions in the 8 mm skin defect by the following method. A molar excess of decontamination solution (approximately 5 ml), with respect to the applied HD, was decanted into the skin defect, creating a contained meniscus of liquid (i.e., HD and decontamination solution). The decontamination solution was allowed to react with the HD for five minutes prior to aspiration of all residual fluid with a pasteur pipette (Baxter Scientific, McGaw Park, IL). Fresh decontamination solution was added to the skin defect and allowed to react for an additional five minutes. This solution was subsequently aspirated, and the animals were contained within a chemical fume hood for 24 hours prior to removal to an animal housing area.

HEMATOLOGY

Guinea pigs were randomly assigned to six individual exposure groups of six animals each (except the HD exposure/No Decontamination group which was comprised of three animals) as outlined in Table 1. One day prior to HD exposure, the animals were anesthetized by carbon dioxide inhalation¹² and a blood sample (0.3 ml) collected by pre-cava venipuncture as described by Bivin¹³ with a 1 ml syringe and 23 gauge needle. These samples were placed in tubes containing ethylenediaminetetraacetate (EDTA) (Microtainer, Becton, Dickinson & Co., Rutherford, NJ) for hematology, and used as each animals' own control blood count.

On day six following exposure/decontamination (exposures were done on day zero), animals were anesthetized by isoflurane inhalation and exsanguinated by open cardiac puncture¹³ saving an aliquot of blood in EDTA-containing tubes for hematology.

QUANTITATIVE ANALYSIS

Clinical hematology assay included: leukocyte count (WBC), erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLAT).

Cellular counts and computed parameters were obtained using an Ortho ELT-8 hematology analyzer (Ortho Diagnostics, Westwood, MA) in accordance with instructions for use and reagents supplied by the manufacturers.

LESION EVALUATION

Visual examination of lesions was conducted at 48 hours post-exposure/decontamination to assess the degree of induced tissue damage. A numerical rating (i.e., Not Affected [0], Mild [1], Moderate [2], or Severe [3]) was determined for each lesion in the following categories of tissue damage: erythema, edema and necrosis. The numerical grades for tissue damage in each of the three categories, for each treatment group, was established by the arithmetic average of individual animal grades. The overall rating of lesion severity was then determined by the numerical summation of individual category grades for each treatment group.

STATISTICAL ANALYSIS

Hematologic parameters of control and post-exposure/treatment groups (n=6) were compared using a two way analysis of variance (ANOVA) with respect to presence of HD, and decontamination solutions employed. A Duncan's multiple range test was used to assess differences between solutions if significant changes were noted at the $p < 0.05$ level in the

WBC count following exposure and decontamination. All tests were made at the alpha = 0.05 level.

RESULTS

Leukocyte Counts

Individual animals did not express a consistent elevation nor depression of their total WBC count (with respect to pre-exposure/decontamination control WBC count) following decontamination with any of the solutions. Further, the positive control (i.e., exposed but not decontaminated) animals did not manifest consistent nor significant changes in WBC count following an intra-wound exposure of 20 mg/kg HD with respect to non-exposed controls.

Table 1 contains mean pre-exposure (control) and post-exposure/decontamination leukocyte counts with differences between samples for each treatment group (\pm SEM).

Decon Sol No HD/HD	Mean Pre-Exp WBC Ct. SEM	Mean 6 Day WBC Ct. SEM	Diff in Mean SEM
No Decon	7.93	5.12	2.82
No HD	0.32	0.41	0.54
No Decon	7.50	7.27	0.23
HD	0.51	1.02	1.07
H ₂ O Decon	9.43	7.03	2.40
No HD	0.80	0.94	0.72
H ₂ O Decon	10.30	9.15	1.15
HD	0.75	1.08	1.13
0.5% NaOCl	9.50	7.88	1.62
No HD	1.13	0.71	1.34
0.5% NaOCl	9.05	9.78	0.73
HD	0.61	0.82	0.82
0.5% CaOCl	8.75	7.22	2.40
No HD	0.47	1.04	0.97
0.5% CaOCl	9.12	6.72	2.40
No HD	0.53	1.24	0.97
2.5% NaOCl	9.48	6.67	2.82
No HD	0.48	0.82	1.16
2.5% NaOCl	8.87	6.67	2.20
HD	1.02	0.99	0.91
2.5% CaOCl	9.65	7.75	1.90
No HD	0.58	0.45	0.89
2.5% CaOCl	9.98	8.87	1.20
No HD	0.42	0.87	1.20

Table 1. Mean WBC count (\pm SEM) pre- and post-HD exposure decontamination. Each group of animals acted as their own control (n = 6, except No Decon/HD where n = 3).

Erythrocyte and Platelet Parameters

No significant changes were observed in erythrocyte or platelet parameters.

Lesion Severity

Animals decontaminated with any solution (including water) manifested visibly more severe lesions than did those exposed but not decontaminated. Immediate post-decontamination bleeding from the wound site was observed in half of the animals (12 of 24 animals) decontaminated with either of the 2.5% hypochlorite solutions. Bleeding was not observed to occur from the animals decontaminated with other solutions. Lesion severity ratings are contained in Table 2.

Lesion Evaluation												
	None			H ₂ O			0.5% NaOCl			0.5% CaOCl		
No	Er	Ed	Nc	Er	Ed	Nc	Er	Ed	Nc	Er	Ed	Nc
HD	1	0.75	1	0.5	0.5	0.5	2.25	1	2.25	1	1	2.1
Total	2.5			1.5			5.5			4.1		
Yes	Er	Ed	Nc	Er	Ed	Nc	Er	Ed	Nc	Er	Ed	Nc
HD	1.5	0.88	0.5	1.5	1.5	2.75	1.25	1.25	1.5	1.5	1	3
Total	2.9			5.3			4.0			5.5		

Table 2. Lesion evaluation 48 hr post-exposure/decontamination procedure (n = 6). Abbreviations: Er = erythema, Ed = Edema, Nc = Necrosis. Numerical grading system: 0 = No Effect, 1 = Slight, 2 = Moderate, 3 = Severe (scores in chart reflect the mean for each group, total is the arithmetic summation).

DISCUSSION

Since the night of July 12, 1917, when Germany launched a chemical attack against French troops in Belgium¹⁴, HD has been a chemical warfare (CW) agent of importance. In the face of 76 years of subsequent research, several hypotheses have been proposed to elucidate the mechanism of HD toxicity.^{14,15,16} Still no definitive therapeutic agents have been found to combat toxicity once the agent is absorbed. As such, the first and most rational approach for the prevention of HD injury is to block its absorption by physical barrier, or ensure its rapid removal (by decontamination) prior to dermal absorption.¹⁷

The ideal decontaminant for HD or any other CW agent would consist of materials that are capable of rapid reaction with, or sorption of the chemical agent, have little or no inherent toxicity, be inexpensive, and not cause a great logistical burden.³ At present there is no single chemical decontamination compound or system that is considered safe or universally efficacious for all CW agents.^{18,19} This research attempted to establish the optimal strength and type of hypochlorite solution (a class of solutions currently fielded by the U.S. military) to ameliorate HD toxicity and more closely approach the characteristics of an ideal decontaminant.

Sulfur mustard, a β -chloroethyl vesicant, is a cytotoxic agent capable of killing cells in a dose-dependent manner. Specifically, the blood-forming cells are among the most sensitive to intoxication.²⁰ Anslow and Houck (1946) asserted that induced leukopenia (i.e., WBC

suppression) affords a good index of the severity of intoxication due to sulfur mustard exposure.²¹ Gold and co-workers established that 20 mg/kg HD given subcutaneously induces a significant, maximal peripheral leukopenia (66.5% reduction from control) on the sixth day following exposure in the EHGP.¹⁰ For these reasons, we proposed the examination of induced peripheral leukocyte suppression (on the sixth day post-exposure), as a means of determining the severity of HD intoxication following exposure and subsequent decontamination in the wounded EHGP.

The rate of chemical inactivation (e.g. hydrolysis) of HD by various solutions¹⁴, the rate of evaporation of HD^{16,17}, and the rate of topical absorption have all been studied as independent phenomena in an *in vitro* system. Few researchers,⁵ though, have considered them together in a biologically complex animal model system. Korte *et al* attempted to fill this gap in vital information with an animal model study that employed dermal lesion area as a marker of decontamination efficacy.

Hydrolysis of HD in water is relatively rapid at vital temperatures (e.g., 2.6 minutes at 37.4 degrees Celsius, and 0.72 minutes at 40 degrees Celsius¹³) and can be expected to be more rapid in the face of a potent hydrolyzing agent such as sodium or calcium hypochlorite.¹⁴

Several researchers^{16,17} have studied the penetration of HD through intact skin and found that maximal absorption is obtained at 5 minutes. Renshaw determined that absorption and tissue binding by intact skin is complete within two minutes.¹⁶ He also found that HD penetrates human skin at a rate of 1-4 $\mu\text{g}/\text{cm}^2/\text{min}$ at 21° after exposure to either liquid or saturated vapor.¹⁶

Renshaw and Collumbe both suggested that only 20% of small applied doses of HD will be absorbed by intact skin at standard room temperature and humidity. The remaining 80% of applied HD is therefore, expected to evaporate.^{16,17}

Understanding the rates of absorption and evaporation, one would conclude that each is rapidly completed and continues until residual HD is either absorbed, evaporated, or removed by some other means. As such, we decided that the ideal timeframe within which to initiate decontamination was the two minutes; the period suggested to allow submaximal absorption and evaporation. Our decontamination procedure was biphasic so that we could augment chemical inactivation of HD with physical removal, a process called for in field decontamination.⁴ We chose to decontaminate for two periods of five minutes each so that an effective time is allotted for hydrolysis, based on *in vitro* data.¹³

Sulfur mustard had a direct inhibitory effect on the blood-forming cells²⁰. Tissue destruction and repair with concomitant inflammation, on the other hand, subjects the animal to a complex cascade of events that greatly increases the level of WBC in the peripheral circulation.²² In consequence, a balance, somewhere between maximum and minimum was struck between inflammation-induced leukocytosis and HD-induced leukopenia.

The results of our WBC counts did not determine the ideal hypochlorite solution for use in decontaminating HD-exposed wounds. In fact, we found no consistent changes in WBC count resulted in any of the treatment groups. We suggest that factors such as: wound inflammation, and the interplay of evaporation and absorption from the wound, modified peripheral WBC count so that no consistent, significant HD-induced WBC suppression was observed.

We expected contaminated lesions to be less severe than undecontaminated lesions. We found that lesions decontaminated with any solution manifested visibly more severe lesions than those not decontaminated.

Many factors were considered to result in the levels of tissue damage observed. We consider the direct dermotoxic nature of hypochlorite solutions, the flushing of liquid from the wound sight, and the contact of chemical break-down products of HD all significant in the degree of lesion severity. Direct HD dermal injury was probably not as significant based on the

finding that undecontaminated, but exposed, animals manifested the least severe lesions.

Direct tissue trauma by hypochlorite solution was a significant cause of lesion severity, based on the finding that unexposed, hypochlorite-decontaminated animals manifested more severe lesions than non-exposed, undecontaminated animals. Lesions decontaminated with these solutions also manifested more severe lesions than did water decontaminated, non-exposed animals. Hypochlorite decontamination solutions were alkaline in pH and may be considered to be directly damaging to cells during decontamination of an open wound.

Byproducts of the chemical hydrolysis of HD, whether formed by interaction with hypochlorite or water, were probably the most offending agent, based on our finding that similar lesion severity following decontamination was obtained with either water or hypochlorite solutions.

Bleeding from some lesions as well as excessive applied fluid volume may have had a role in mechanically flushing HD or its hydrolysis products from the initial site of exposure. This may have increased the size of the observed lesion, but not the lesion severity as it was of consistent severity with those lesions where bleeding or fluid overflow were not observed.

Lesion-associated inflammation appeared to play a major role in the total observed peripheral WBC count, confounding its use as a marker of total absorbed HD. Lesion severity, as well, was shown to be a poor marker of the total systemically-absorbed HD dose. This study showed that decontaminating HD-exposed lesions exacerbated their severity but did not make conclusions about total absorbed HD. Resultantly, one should not use this study to suggest that persons not decontaminate HD exposed wounds. Fore in not decontaminating, animal lethality, by systemic toxicity, may be a result of an attempt to reduce lesion severity.

We must conclude that peripheral WBC counts are poor biomarkers of decontamination efficacy in the HD-exposed, wounded EHGP model. Had we been able to measure the total absorbed HD dose more definitively we could have better determined the efficacy of decontamination in an objective manner. We hold promise of the future validation of the HD-exposed wounded EHGP model, perhaps by using radioactively-labeled HD or measuring end products of systemic HD metabolism (e.g., thidylglycol)²³, because it takes into consideration all of the biological interactions associated with a live animal model in a species currently employed in dermal HD toxicity work.^{6,7}

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